PROTEIN CHANGES IN DEVELOPMENT [1,2]

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Biologists have long been in agreement with the thesis that morphological differentiation is either preceded by, or accompanied simultaneously by, an underlying chemical differentiation. In the testing of this hypothesis, considerable attention has been focused on the qualitative and quantitative changes occurring in the proteins of developing embryos.

The usual biochemical techniques have been employed to describe and to quantitate these changes. One should mention the solubility studies of sea urchin embryo proteins by Mirsky (1936), the electrophoresis of sea urchin embryo proteins by Monroy (1950), and the electrophoresis of amphibian embryo proteins by Flickinger and Nace (1952). Recently the serological technique has been used quite extensively; the precipitin test by Cooper (1948), Ebert (1951), Harding *et al.* (1955); the Oudin technique by Spar (1953) and by Cooper (1948).

In addition to these *in vitro* studies a number of *in vivo* studies have been carried out. Ebert (1953, 1955) and his co-workers have studied the effects of specific antisera on living chick embryos, and Tyler and Brookbank (1956) have studied the cytotoxic effects of specific antisera on sea urchin eggs. We have carried out similar studies on dissociated sponge cells (Spiegel, 1955). The results of these investigations have been extensively summarized by Nace (1955) and by Tyler (1957).

Briefly, the results obtained through the use of the serological techniques on amphibians and sea urchins are as follows. In the sea urchin, it has been demonstrated by Perlmann and Gustafson (1948) and Perlmann (1953) that new antigens appear during development. These new antigens were first detected in the gastrula stage. Harding *et al.* (1954, 1955), working with lethal hybrid sea urchin embryos, were able to detect paternal antigens at the blastula stage.

In the frog, it seems fairly well established from the work of Cooper (1946, 1948, 1950) and of Spar (1953) that new antigens are found in the blastula and gastrula stages. Ten Cate and Van Doorenmaalen (1950) and Flickinger *et al.* (1955) have shown that there is good correlation between the time of appearance of lens antigen and of the lens itself. Flickinger and Nace (1952) have detected new antigens in the tail-bud stage.

Clayton (1953) has further demonstrated that between the blastula and gastrula stage, before neurulation, and between neurulation and the formation of the tail-bud, a synthesis of antigenic material occurs. Ectoderm and archenteron roof contain fractions specific to themselves as well as having common antigens.

A word of caution seems warranted when considering these results. It should be mentioned that Tyler (1957) has pointed out that in investigations of this kind (in which antisera produced against saline extracts—after suitable absorptions—have been used to detect changes in antigens) there is the uncertainty as to whether or not a particular antigenic structure remains associated with a saline-soluble constituent and as to whether or not it is available for reaction in specific absorption procedures. The appearance of new antigens or the loss of old antigens may represent a change in solubility, rather than the synthesis or destruction of an antigen. The above evidence, therefore, which has been represented as a synthesis of new material may rather reflect changes in solubility of pre-existing substances. This point will be more fully considered in the discussion of the results presented in this paper.

The study to be reported here involved the use of the technique of zone electrophoresis to follow the changes occurring in the proteins of developing embryos and to compare these changes with adult organ proteins.

Materials and Methods

I. *Preparation of extracts*

A. *Developmental stages.* Embryos of the frog, *Rana pipiens,* from the unfertilized egg through stage 21 (Shumway, 1940) were used. Eggs were obtained by pituitary injection of large adult females and fertilized by the usual method of stripping directly into a suspension of macerated testes in 10% Holtfreter's solution, pH 7.8. The embryos were then washed three times with sterile 10% Holtfreter's solution, pH 7.8, and incubated at 19–20° C. until the desired stage was attained. Extracts were prepared from embryo populations in which at least 95% of the embryos were of the same stage and developing normally. Regardless of the stage at which the extract was made, 100–200 embryos of each population were always carried through stage 22 and if subsequent development of these controls showed more than 5% of the embryos to be abnormal, the extract was discarded. For the unfertilized egg extracts, 100–200 eggs were fertilized and incubated as described above. If more than 5% of the embryos were abnormal, the extract was discarded.

The jelly of individuals of the desired stage was then removed by the papain-thioglycolate method of Spiegel (1951). After washing five times with sterile 10% Holtfreter's solution, pH 7.8, 500–1000 jelly-free embryos or eggs were allowed to settle out, under gravity, in a 50-ml. cellulose nitrate centrifuge tube. Excess supernatant fluid was removed by aspiration, leaving only the small amount of solution that was trapped in the interstices between individuals. The loosely packed embryos were frozen-thawed, homogenized in a glass homogenizer, and centrifuged at 23,000 g for 10 minutes. The clear supernatant fluid minus the fat layer was removed by aspiration and stored at − 20° C. until used. All developmental stages were treated in identical fashion. The temperature throughout the extraction procedure was 4° C.

B. *Adult organs.* The following organs were dissected from adult frogs of both sexes: brain, gastrocnemius muscle, and small intestine. These organs were then extracted by the identical method used for developmental stages. Adult blood

was obtained by cardiac puncture from adult male and female frogs, diluted with an equal amount of 10% Holtfreter's solution, pH 7.8, centrifuged at 23,000 g for 10 minutes, the supernatant fluid, strongly colored by hemoglobin, removed by aspiration, and stored at $- 20°$ C. until used.

II. *Electrophoresis*

Before electrophoresis, the extracts were thawed and dialyzed in the cold vs. three changes of the electrophoretic buffer, Veronal, pH 8.6, $\mu = 0.05$, for 24 hours. The dialysates were centrifuged at 23,000 g for 10 minutes and 0.02 ml. of supernatant fluid was applied to filter paper strips (Spinco Part No. 300–028). The 8-strip Spinco Durrum-type electrophoresis cell and the Spinco Model R regulated power supply were used. Electrophoresis was carried out at room temperature using 25 ma constant current for 6 hours. Under these conditions rather remarkable reproducibility was obtained. Each stage or adult organ extract was prepared a minimum of five times and each preparation subjected to at least three separate runs, making a total of at least 15 electrophoretic runs per type of extract.

III. *Staining and scanning of paper strips*

At the completion of the run, the papers were dried at 115° C. for 30 minutes and stained by the rapid brom phenol blue procedure for proteins described by Durrum (1958). The strips were rinsed in methanol for 6 minutes, stained for 30 minutes in 0.1% brom phenol blue in methanol, followed by three rinses in 5% acetic acid of 6 minutes duration per rinse. They were then blotted, dried at 115° C. for 15 minutes, exposed to concentrated ammonium hydroxide vapor for 15 minutes, and scanned with the Photovolt Densitometer Model 525 (using a 505-millimicron broad band filter) coupled to the Varicord Variable-Response Recorder. For glycoprotein identification, the periodic acid-fuchsin sulfite method of Köiw and Gronwall (1952), as described by Durrum (1958), was used. The strips were scanned as described above.

RESULTS

A. *Developmental stage extracts*

The brom phenol blue protein pattern obtained with unfertilized egg extracts is shown in Figure 1. Seven cation-bands and two anion-bands were noted. We chose to use the term bands rather than proteins since the absence of additional data such as electrophoresis at pH's other than 8.6, salt fractionation, etc., did not allow us to conclude that each band represented a single protein. Indeed, careful examination of the densitometer records often revealed that a major band was composed of two or more small peaks. For example, the cation-band labelled B in Figure 1 was actually composed of three smaller peaks, readily reproducible from run to run. In addition to these stained bands there was an additional band, labelled *fl,* which was not stained by brom phenol blue but which charred as a result of drying at 115° C. for 30 minutes and appeared as a yellow-brown band in the unstained strip. It was the only band which fluoresced under ultraviolet illumination. It did not correspond to any of the brom phenol blue-stained bands
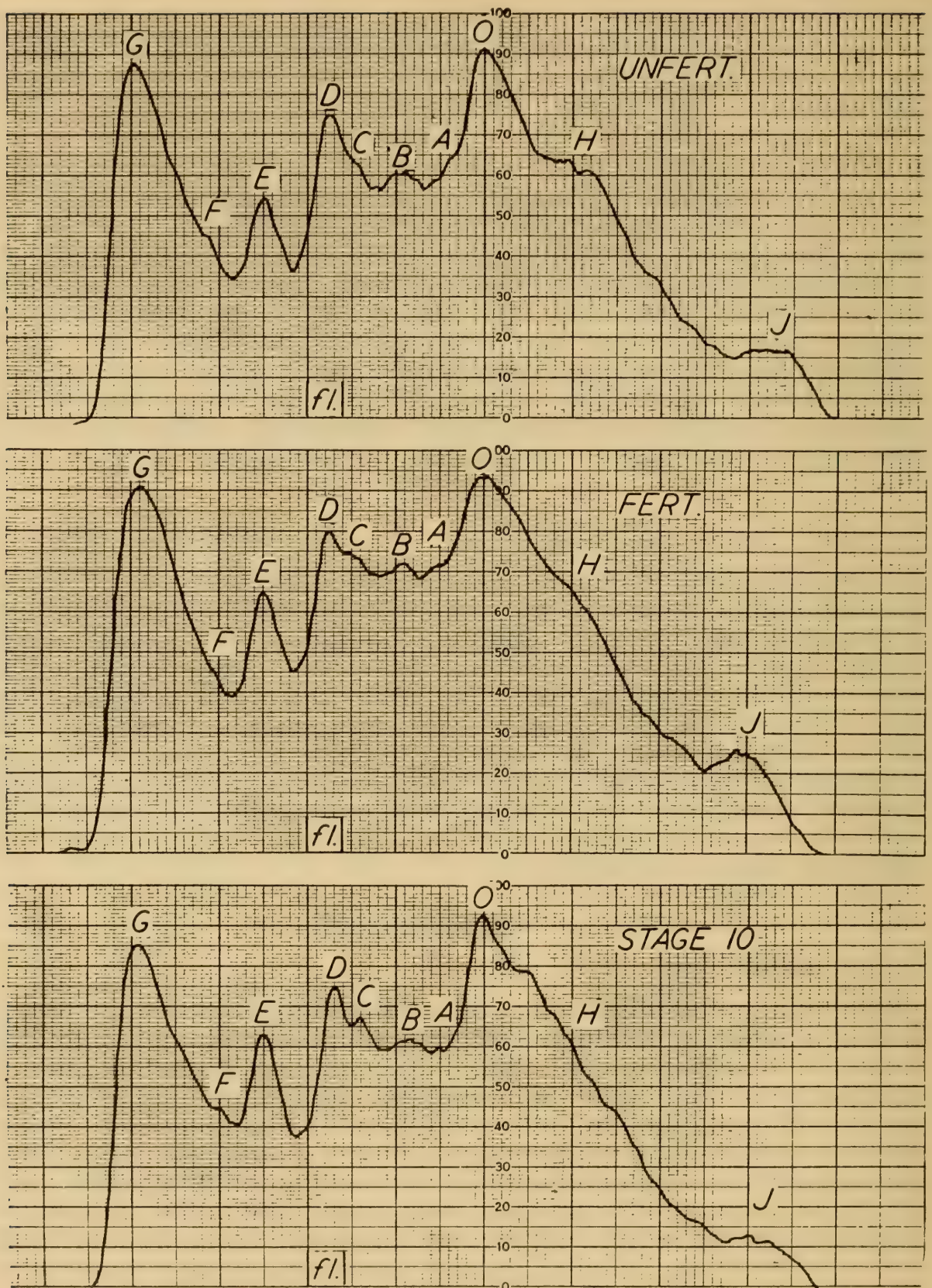
FIGURE 1. Densitometer tracings of brom phenol blue-stained paper strips of developmental stage extracts after electrophoresis. Ordinate labelled 0 represents point of application. Values 0–100 on the 0 ordinate represent the relative density of brom phenol blue stain. To the right of the 0 ordinate are the anions; to the left are the cations. Abscissa units represent relative distance away from the point of application.
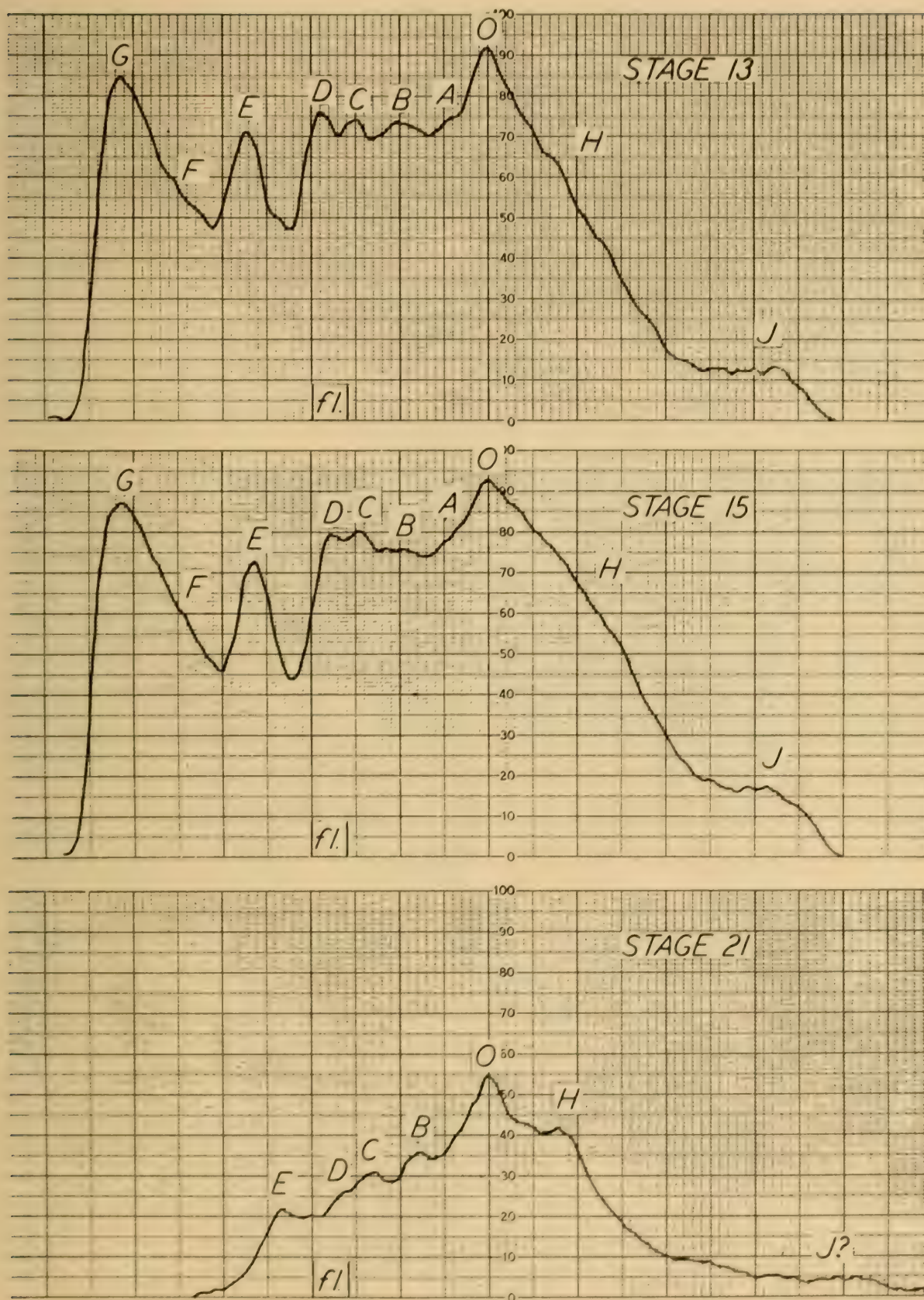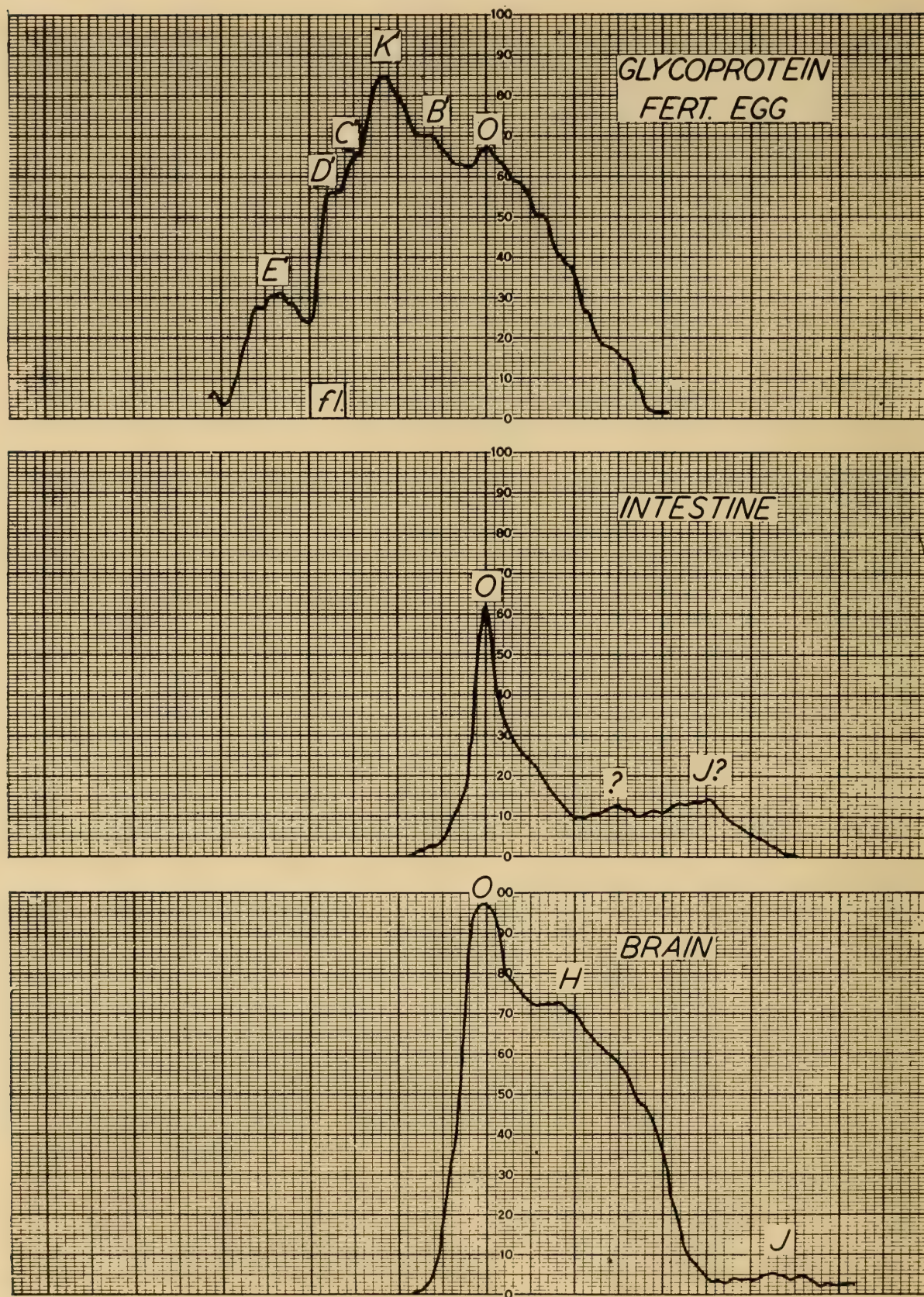
FIGURE 2. Densitometer tracings of brom phenol blue-stained paper strips of developmental stage extracts after electrophoresis. Symbols as in Figure 1.

FIGURE 3. Upper: Densitometer tracings of periodic acid-fuchsin sulfite-stained paper strips of developmental stage extracts after electrophoresis. Middle and bottom: Densitometer tracings of brom phenol blue-stained paper strips of adult organ extracts after electrophoresis. Symbols as in Figure 1.

and its limits were demarcated in the figure by the two lines on either side of the label *fl* in the figures.

The patterns obtained with fertilized egg extracts and stage 10 embryo extracts are shown in Figure 1. It was readily noted that following fertilization there was a decrease in the concentration of the H band which was perhaps comparable to the "Mirsky protein" found in the sea urchin egg. There was also a quite noticeable progressive increase during development in the concentration of the cation-band C. All other bands remained essentially constant in concentration through stage 15 (Figs. 1 and 2).

By stage 21, bands A, F and G were no longer detected (Fig. 2). The presence of band J was questionable and at best was in very low concentration and migrated as a broad band rather than as a sharply defined entity. The over-all protein concentration, under the conditions of extraction and electrophoresis employed, was markedly reduced. Band H, however, increased in relative concentration and by stage 21 approximated the relative concentration found in the unfertilized egg.
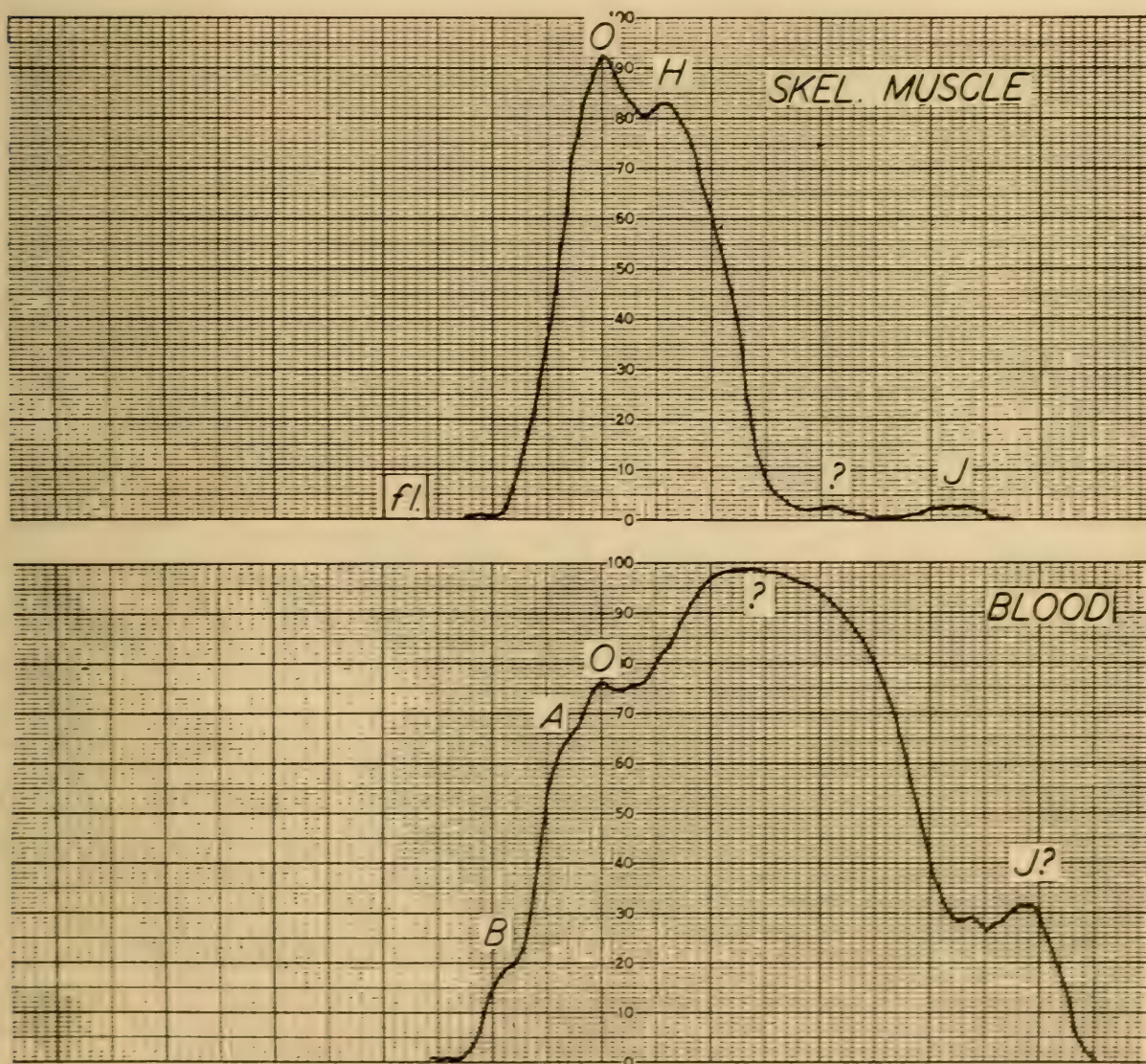

FIGURE 4. Densitometer tracings of brom phenol blue-stained paper strips of adult organ extracts after electrophoresis. Symbols as in Figure 1.

protein patterns of adult organ extracts do exhibit differences. There are some components present in more than a single organ but others are apparently confined to a single organ. For example, bands H and J are present in both skeletal muscle and in brain extracts; the *fl* band, on the other hand, is present only in the skeletal muscle extract.

These patterns suggest that all, or most, of the proteins found in adult organs are first found in the unfertilized egg. As development proceeds some of the cells lose their ability to manufacture one or more particular proteins. Correlated with this loss of ability to synthesize a particular protein is a corresponding morphological differentiation. Differentiation, therefore, could involve a loss of the ability to synthesize a protein rather than the synthesis of a new protein. The work of Ebert (1953, 1955) on the distribution of cardiac myosin in the chick embryo is in support of this hypothesis. Ebert demonstrated that early in development cardiac myosin is rather uniformly distributed throughout the blastoderm. As development proceeds, the ability to synthesize cardiac myosin is eventually restricted to those cells located in the two heart-forming regions.

An alternative to the above explanation is equally probable. The differences observed in the protein patterns of adult organs and those of the embryo may reflect changes in the solubility of the proteins of the unfertilized egg or of adult organs rather than the loss or gain of any synthetic capacity. As a result of this line of reasoning, one could assume that the same proteins are present in the adult brain as in the unfertilized egg, but some of these proteins are insoluble, perhaps under the conditions of extraction, in the adult organ. At present, the more probable hypothesis cannot be determined. It is likely that both processes are involved in differentiation. The solubility and location of the proteins detected in this investigation are unknown. Whether we are dealing with the soluble proteins of the cytoplasm or those proteins soluble in the small amount of 10% Holtfreter's solution (and ultimately in the electrophoretic buffer) used for extraction is not readily apparent. Further studies are necessary to determine location, solubilities, etc. of these proteins. Why the present work does not show the appearance of "new" proteins (proteins insoluble in the unfertilized egg but soluble at later stages)—if the latter hypothesis is correct—cannot be answered at the present time.

It must be emphasized that the identification of proteins in this study has been made solely on the basis of mobility in an electric field. It is possible that new and different proteins from those of the unfertilized egg are being synthesized throughout development. These new proteins could have the same or closely matching mobilities as those of the early embryo, would therefore not be detected as new proteins, and could perhaps give the impression of a static population of protein types which does not exist. Further characterization of these proteins by other biochemical techniques will resolve this question. Of interest is the observation that, under these conditions, the majority of the embryonic proteins are cations; those of the adult are primarily anions. It is possible that those cation-bands which are lost during development represent the yolk proteins and are utilized as an energy source.

If the over-all hypothesis be true, that differentiation can be brought about by, or is associated with, the loss of the ability to synthesize a pre-existing protein

and/or changes in solubilities of pre-existing proteins, one must consider the ever-increasing body of literature apparently demonstrating the presence of new antigens in development. All of these papers, to our knowledge, have dealt solely with saline-soluble antigens. The appearance of a new antigen in development may rather represent a change in solubility rather than a synthesis of new antigenic material. For example, it is entirely possible that an antigen present in the blastula stage is saline-insoluble but in the gastrula stage is soluble in saline. Such an antigen, after the usual absorptions, etc. have been carried out, would then be described as a new antigen arising during gastrulation rather than as a change in solubility of a pre-existing substance. The demonstration by Markert and Møller (1959) of multiple forms of enzymes—the isozymes—which are tissue, ontogenetic, and species-specific can also be explained, rather simply, on the basis of changes in solubility.

The recent paper by Solomon (1959) offers a striking demonstration of changes in location, and therefore of solubility, of a particular enzyme during development. It was shown that the mitochondrial glutamic dehydrogenase of the chick embryo liver increased in activity after the twelfth day of incubation and this rise was followed by a sudden drop of glutamic dehydrogenase activity in the supernatant fluid (0.25 M sucrose) after 15 days of incubation. Supernatant fluid glutamic dehydrogenase activity is four times that of mitochondria at 7 days' incubation; 6 times at 12 days' incubation; $\frac{1}{3}$ times at 20 days' incubation. The thesis that changes in the intra-cellular distribution of enzymes may occur during differentiation has been put forth by Krahl (1950), and Gustafson (1954).

The work of Schechtman (1955) and Nace (1953) has further demonstrated that the serum proteins of the chick are present in the unincubated egg and are probably transferred from the maternal circulation to the egg.

The results obtained using the periodic acid-fuchsin sulfite staining technique for glycoproteins remain to be considered (Fig. 3). The band K' does not correspond to any of the brom phenol blue-stained bands and is probably a polysaccharide. The fact that the remaining four cation-bands, B', C', D' and E', correspond in position to the B, C, D and E bands, respectively of the brom phenol blue-stained strips, coupled with the observation that the periodic acid-fuchsin sulfite-stained bands were detected in early stages but not in stage 21 extracts, has led to the following interpretation.

It is possible that these bands represent a polysaccharide migrating at the same rate as proteins composing the band, or they may represent a glycoprotein. By stage 21, synthesis of these polysaccharides or glycoproteins has ceased or their solubility has changed. It would therefore be justifiable to label these bands as distinct from other proteins present in the band. It is also possible that the periodic acid-fuchsin sulfite technique is less sensitive than the brom phenol blue technique. Due to the decrease in protein concentration of the stage 21 extracts (Fig. 2) a positive brom phenol blue test but negative polysaccharide test results. Which of these interpretations is correct remains to be determined. The labelling of these bands with prime letters indicates the possibility of their being discrete entities.

It is realized that the present paper by no means offers conclusive proof, but merely suggests that differentiation may, in part, involve the loss of ability to synthesize a particular protein and/or changes in the solubility of pre-existing

proteins. The intra-cellular locations, concentrations, tissue distributions, and further biochemical characterizations of these proteins are being carried out in this laboratory at the present time.

Summary

1. Extracts of developmental stages and of four adult organs of the frog, *Rana pipiens,* were examined by the technique of paper electrophoresis.

2. Seven protein cation-bands and two protein anion-bands stained with brom phenol blue were detected in extracts of developmental stages from the unfertilized egg through stage 15. By stage 21, two of the cation-bands were no longer detected.

3. Examination of the adult organ extracts revealed organ-specific proteins in addition to proteins common to all organs tested.

4. The results were interpreted in terms of the hypothesis that differentiation involves, in part, the loss of ability to synthesize a particular protein(s) and/or changes in the solubility of pre-existing proteins.

LITERATURE CITED

CLAYTON, R. M., 1953. Distribution of antigens in the developing newt embryo. *J. Embryol. Exp. Morphol.,* 1: 25–42.

COOPER, R. S., 1946. Adult antigens (or specific combining groups) in the egg, embryo, and larva of the frog. *J. Exp. Zool.,* 101: 143–172.

COOPER, R. S., 1948. A study of frog egg antigens with serum-like reactive groups. *J. Exp. Zool.,* 107: 397–437.

COOPER, R. S., 1950. Antigens of frog embryos and of adult frog serum studied by diffusion of antigens into agar columns containing antisera. *J. Exp. Zool.,* 114: 403–420.

DURRUM, E. L., 1958. Paper electrophoresis. *In:* A Manual of Paper Chromatography and Paper Electrophoresis, by R. J. Block, E. L. Durrum and G. Zweig. Academic Press Inc., Publishers, New York, N. Y. Pages 489–667.

EBERT, J. D., 1951. Ontogenetic change in the antigenic specificity of the chick spleen. *Physiol. Zööl.,* 24: 20–41.

EBERT, J. D., 1953. An analysis of the synthesis and distribution of the contractile protein, myosin, in the development of the heart. *Proc. Nat. Acad. Sci.,* 39: 333–344.

EBERT, J. D., 1955. Some aspects of protein biosynthesis in development. *In:* Aspects of Synthesis and Order in Growth, D. Rudnick, editor. Princeton University Press, Princeton, N. J. Pages 69–112.

FLICKINGER, R. A., AND G. W. NACE, 1952. An investigation of proteins during the development of the amphibian embryo. *Exp. Cell Res.,* 3: 393–405.

FLICKINGER, R. A., E. LEVI AND A. E. SMITH, 1955. Some serological experiments relating to the embryonic development of the lens. *Physiol. Zööl.,* 28: 79–85.

GUSTAFSON, T., 1954. Enzymatic aspects of embryonic differentiation. *Intern. Rev. Cytol.,* 3: 277–327.

HARDING, C. V., D. HARDING AND P. PERLMANN, 1954. Antigens in sea urchin hybrid embryos. *Exp. Cell Res.,* 6: 202–210.

HARDING, C. V., D. HARDING AND I. W. BAMBERGER, 1955. On cross fertilization and the analysis of hybrid embryonic development in echinoderms. *Exp. Cell Res.,* suppl., 3: 181–187.

KÖIW, E., AND A. GRONWALL, 1952. Staining of protein-bound carbohydrates after electrophoresis of serum on filter paper. *Scand. J. Clin. and Lab. Invest.,* 4: 244–246.

KRAHL, M. E., 1950. Metabolic activities and cleavage of eggs of the sea urchin, *Arbacia punctulata. Biol. Bull.,* 98: 175–217.

MARKERT, C. L., AND F. MØLLER, 1959. Multiple forms of enzymes: tissue, ontogenetic, and species specific patterns. *Proc. Nat. Acad. Sci.,* 45: 753–763.

MIRSKY, A. E., 1936. Protein coagulation as a result of fertilization. *Science,* **84**: 333–334.

MONROY, A., 1950. A preliminary electrophoretic analysis of proteins and protein fractions in sea urchin eggs and their changes on fertilization. *Exp. Cell Res.,* **1**: 92–104.

NACE, G. W., 1953. Serological studies of the blood of the developing chick embryo. *J. Exp. Zool.,* **122**: 423–448.

NACE, G. W., 1955. Development in the presence of antibodies. *Annals N. Y. Acad. Sci.,* **60**: 1038–1055.

PERLMANN, P., AND T. GUSTAFSON, 1948. Antigens in the egg and early developmental stages of the sea urchin. *Experientia,* **4**: 481–482.

PERLMANN, P., 1953. Soluble antigens in sea urchin gametes and developmental stages. *Exp. Cell Res.,* **5**: 394–399.

SCHECHTMAN, A. M., 1955. Ontogeny of the blood and related antigens and their significance for the theory of differentiation. *In:* Biological Specificity and Growth, E. G. Butler, editor. Princeton University Press, Princeton, N. J. Pages 3–31.

SHUMWAY, W., 1940. Stages in the normal development of *Rana pipiens.* I. External form. *Anat. Rec.,* **78**: 139–147.

SOLOMON, J. B., 1959. Changes in the distribution of glutamic, lactic and malic dehydrogenases in liver cell fractions during development of the chick embryo. *Dev. Biology,* **1**: 182–198.

SPAR, I., 1953. Antigenic differences among early developmental stages of *Rana pipiens. J. Exp. Zool.,* **123**: 467–497.

SPIEGEL, M., 1951. A method for the removal of the jelly and vitelline membrane of the egg of *Rana pipiens. Anat. Rec.,* **111**: 544.

SPIEGEL, M., 1955. The reaggregation of dissociated sponge cells. *Annals N. Y. Acad. Sci.,* **60**: 1056–1078.

TEN CATE, G., AND W. S. VAN DOORENMAALEN, 1950. Analysis of the development of the eye-lens in chicken and frog embryos by means of the precipitin reaction. *Konink. Med. Akad. Wetenschap.,* **53**: 894–909.

TYLER, A., 1957. Immunological studies of early development. *In:* The Beginnings of Embryonic Development. Amer. Assoc. Adv. of Sci., Washington, D. C. Pages 341–382.

TYLER, A., AND J. W. BROOKBANK, 1956. Antisera that block cell division in developing eggs of sea urchins. *Proc. Nat. Acad. Sci.,* **42**: 304–308.